intellectuelle du Canada
Un organisme d'Industrie Canada

Intellectual Property Office
An Agency of Industry Canada

15 SEP 2000 (15 09 00)

REC'D 26 SEP 2000
WIPO

Bureau canadien des brevets

Certification

La présente atteste que les documents ci-joints, dont la liste figure ci-dessous, sont des copies authentiques des documents déposés au Bureau des brevets.

Canadian Patent Office

Certification

This is to certify that the documents attached hereto and identified below are true copies of the documents on file in the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,284,103,** on September 30, 1999, by **THE GOVERNING COUNCIL OF THE UNIVERSITY OF TORONTO,** assignee of Jane Aubin and Edith Bonnelye, for "Estrogen Related Receptor, ERR, A regulator of Bone Formation".

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

L. Rejimbal
Agent certificateur/Certifying Officer

September 15, 2000
Date

Canada

(CIPO 68)

OPIC CIPO

Estrogen [redacted]ed Receptor, ERRα , A Regulator of [redacted] Formation

Field of the Invention

The present invention relates to the orphan estrogen related receptor, ERRα, which is involved in the regulation of bone formation

Background of the Invention

Nuclear receptors are transcription factors involved in various physiological regulatory processes The superfamily to which nuclear receptors belong comprises both ligand-dependent molecules such as the steroid hormone-, thyroid hormone-, retinoic acid- and vitamin D-receptors, and an increasing number of so-called orphan receptors for which no ligand has yet been determined (Gronemeyer H, Laudet V., 1995, Enmark and Gustafsson ,1996) Indeed, it is not yet known whether the orphan receptors have ligands that await identification or whether they act in a constitutive manner. The orphan receptors display the same structural organization as do the classic ligand-dependent receptors: the A/B domain located in the N-terminal part of the protein harbors a ligand-independent transactivation function (AF-1), the C domain, which is the most conserved part of the molecule, is responsible for the specific DNA-binding activity; the E domain contains the ligand binding hydrophobic pocket and contributes to receptor dimerization and to the ligand-dependent transactivation function (AF-2).

Two orphan receptors, estrogen-related receptor α (ERRα) and ERRβ (Giguere et al , 1988; NR3B1 and NR3B2, respectively, according to the Nuclear Receptors Nomenclature Committee, 1999) are closely related to the estrogen receptors ERα and ERβ (Green et al., 1986, Kuiper et al , 1996; NR3A1 and NR3A2 respectively). ERRα and ERRβ were identified by low-stringency screening of cDNA libraries with a probe encompassing the DNA-binding domain of the human estrogen receptor (ER). Sequence alignment of ERRα and the ERs reveals a high similarity (68%) in the 66 amino acids of the DNA-binding domain and a moderate similarity (36%) in the ligand-binding E

domain, which may explain the fact that ERRα does not bind estrogen. ERRα has been identified as a regulator of the SV40 major late promoter during the early-to-late switch of expression (Wiley et al., 1993) and as a regulator of fat metabolism (Sladek et al., 1997). Yang et al. also showed that ERRα modulates the activating effect of estrogens on the lactoferrin promoter and suggested that ERRα may interact with ERs through protein-protein interaction (Yang et al., 1996)

An anabolic effect of estrogens on bone homeostasis has been documented in post-menopausal osteoporosis (for review see Pacifi, 1996), where bone loss can be retarded reversed by administration of natural or synthetic estrogens. However, the molecular mechanisms by which these compounds act is still poorly understood, as is the nature of their cellular target(s) Bone is a tissue undergoing constant renewal, a remodelling process controlled by osteoblasts that form bone and osteoclasts that resorb it. Although the ERs are expressed at relatively low levels in osteoblasts and even lower levels in osteoclasts (Turner et al., 1994), estrogens have been found to elicit effects ranging from modulation of gene expression to regulation of proliferation in both cell types. Due to their homology to the ERs, we hypothesized that the ERRs may intervene in the signals induced by estrogen in bone. ERRβ expression, however, is restricted to early development and to a few adult tissues (Giguere et al., 1988; Pettersson et al., 1996). In contrast, ERRα has a broader spectrum of expression, including fat, muscle, brain, testis and skin (Bonnelye et al. 1997b). Strikingly, ERRα is also highly expressed in the ossification zones of the mouse embryo (in long bones, vertebrae, ribs and skull), and is more widely distributed in osteoblast-like cells than is ERα(Bonnelye et al., 1997a). Morever it has been shown that ERRα positively regulates the osteopontin gene (Vanacker et al, 1998), an extracellular matrix molecule secreted by osteoblasts and thought to play a role in bone remodelling (Denhardt and Guo, 1993), especially resorption (ref).

Given these observations, we sought to assess more directly whether ERRα plays a functional role in bone formation. The present invention demonstrates that ERRα is more highly expressed in differentiating osteoblastic cells than either ERα and ERβ and that it is differentially regulated during the developmental sequence. The present invention also demonstrates that upregulation of ERRα levels stimulates bone formation while downregulation inhibits bone formation. Our data suggest that modulation of ERRα levels may be a useful therapeutic tool in diseases of bone metabolism

Summary of the Invention

The present invention is directed to the orphan estrogen related receptor, ERRα, expressed by osteoblastic cells which is a potentially important regulator of bone formation and maintenance of bone mass. It has been found that ERRα is differentially expressed throughout all osteoblastic differentiation stages *in vitro*. ERRα is much more highly expressed in osteoblastic cells than either ERα or ERβ. ERRα is expressed more highly in cuboidal osteoblastic cells than in surrounding non-nodular/fibroblastic cells and nuclear expression of ERRα increased as osteoblasts matured. Immunocytochemistry also showed that ERRα and ERβ but not ERα are highly expressed in the developing 21d fetal rat calvaria in sutural cells and cells at the osteogenic front. On the other hand, little ERβ, but abundant ERRα and ERα, are expressed in mature osteoblasts and osteocytes in compact and remodeling bone.

Blocking ERRα expression in either proliferating or differentiating RC cell cultures was found to inhibit cell growth and also demonstrated a proliferation-independent complete inhibition of both mineralized and unmineralized bone nodule formation. Overexpression of ERRα with transient transfection of RC cells resulted in a dose dependent biphasic effect.

Taken together, the findings show that ERRα is more highly expressed in osteoblastic cells than either ERα or ERβ, that ERRα is differentially regulated during the developmental sequence, and that the three receptors are differentially expressed in different cohorts of osteoblasts.

It is therefore apparent that ERRα plays a physiological role in bone formation at both proliferation and differentiation stages. This indicates a therapeutic role for this nuclear transcription factor in various clinical conditions such as osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, peridontal disease, tumoral calcinosis and dermatomyotsitis

According to an aspect of the present invention is the use of ERRα in the regulation of bone formation in a mammal.

According to another aspect of the present invention is the use of the ERRα for the treatment of diseases of bone metabolism.

According to a further aspect of the present invention is the use of ERRα for the treatment of a condition selected from the group consisting of osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, peridontal disease, tumoral calcinosis and dermatomyotsitis.

According to yet a further aspect of the present invention is the use of ERRα for the regulation of bone formation during fetal development and also in adult mammals.

According to still a further aspect of the present invention is the use of ERRα for stimulating the differentiation of osteoblasts from primitive progenitor cells to mature osteoblasts and osteocytes *in vitro* and *in vivo*.

The present invention also provides a therapeutic composition, comprising a purified ERRα as active ingredient, and a method for the treatment of various clinical conditions involving abnormal bone formation. In particular, the composition and method can be used to treat osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, peridontal disease, tumoral calcinosis and dermatomyotsitis.

In accordance with one aspect of the invention there is provided a method for the treatment of a bone developmental disorder in a mammal comprising administering to a mammal an effective amount of a composition which comprises as an active ingredient ERRα in a mixture with a pharmaceutically acceptable diluent or carrier. It is also acknowledged that the ERRα receptor can also be used to regenerate tissues after tissue damage, namely in bone.

In accordan[redacted] a further embodiment, the ERRα sign[redacted] pathway may be modulated by modulating the binding of the ERRα to an ERRα binding partner. Such a binding partner may include for example the estrogen receptor. ERRα can be used to upregulate the transcription and thus expression of certain genes which work together with ERRα to affect skeletal development.

The invention further provides methods for screening candidate compounds to identify those able to modulate signaling by ERRα through a pathway involving ERRα.

For example, the invention provides screening methods for compounds able to bind to ERRα which are therefore candidates for modifying the activity of ERRα. Various suitable screening methods are known to those in the art, including immobilization of ERRα on a substrate and exposure of the bound ERRα to candidate compounds, followed by elution of compounds which have bound to the ERRα.

The invention also provides a method of modulating a ERRα signaling pathway by increasing or decreasing the availability of ERRα or by modulating the function of the ERRα.

The invention further provides methods for preventing or treating diseases characterised by an abnormality in an ERRα signaling pathway which involves ERRα, by modulating signaling in the pathway.

In a further embodiment, a normal version of ERRα, could be provided by gene therapy to restore function in a disease wherein ERRα, is mutated or non-functional.

According to a further aspect of the present invention, transgenic animal models containing a mutant ERRα or alternatively have been developed as a ERRα gene knock out can be used in order to further characterize the role of ERRα in skeletal development. Such a transgenic system also allows for screening of candidate compounds as therapeutics. For example, knock out animals, such as mice, may be produced with deletion of a ERRα gene. These animals may be examined for phenotypic changes and used to screen candidate compounds for effectiveness to reverse these changes.

According to a further aspect of the present invention ERRα expression can be used to target and identify genes activated during particular osteoblast developmental time windows.

In accordance with another aspect of the present invention is a method for screening a candidate compound for effectiveness as an antagonist of ERRα comprising.

(a) providing an assay method for determining a biological activity of ERRα; and

(b) determining the biological activity of ERRα in the presence or absence of the candidate compound, wherein a reduced biological activity in the presence of the candidate compound indicates antagonist activity of the compound.

In accordance with another aspect of the present invention is a method for treating in a mammal a disorder associated with an undesired biological activity of ERRα comprising administering to the mammal an effective amount of a substance selected from the group consisting of

(a) an ERRα antagonist,

(b) an antibody which binds specifically to ERRα, and

(c) an antisense strand comprising a nucleic acid sequence complementary to the sequence or fragment of the sequence and capable of hybridizing to the nucleic acid sequence encoding ERRα; and

(d) an agent which down regulates the expression of the ERRα gene encoding for ERRα.

According to another aspect of the present invention is a method for suppressing in a mammal, the proliferation of a cell capable of being stimulated to proliferate by ERRα, the method comprising administering to the mammal an effective amount of a ERRα antagonist or an antibody which binds specifically to ERRα. Such cells would include but not be limited to primitive progenitor cells, osteoblasts and osteocytes.

Brief Description of the Drawings

The present invention will be further understood from the following description with reference to the Figures, in which

Figure 1 shows a northern blot in which ERRα mRNA from rat calvaria cells grown in the presence and absence of dexamethasone is differentially expressed during osteoprogenitor cell differentiation to mature osteoblasts and osteocytes;

Figure 2 shows ERRα mRNA expression in osteoblast lineage cells at all development times selected on the basis of molecular phenotype of osteopontin, bone sialoprotein, alkaline phosphatase and osteocalcin expression;

Figure 3 shows the expression of ERRα compared with that of ERα and ERβ in RC cell cultures in the presence and absence of dexamethasone;

Figure 4 shows immunocytochemistry using appropriate antibodies and antiserum of RC cells showing different expression patterns of ERRα, ERα and ERβ over time from day 2 to day 15;

Figure 5 shows immunocytochemistry of 21 day fetal rat calvaria sections showing different expression patterns of ERRα, ERα and ERβ;

Figure 6 shows a dose dependent decrease in RC cell number using antisense oligonucleotide specific for ERRα;

Figure 7 shows the effect of antisense oligonucleotide treated RC cells from day 6 of cell culture on nodule formation;

Figure 8 shows the overexpression of ERRα in transiently transfected RC cells on the formation of mineralized bone nodules; and

Figure 9 shows the effect of estrogen and vitamin D_3 on ERRα levels in RC cell cultures.

Detailed Description of the Preferred Embodiments

In accordance with the present invention, it has now been demonstrated that ERRα plays a role in the regulation of bone formation and as such has several potential therapeutic uses especially in the treatment of diseases involving altered bone metabolism ERRα has potential therapeutic use in the treatment of various diseases such as but not limited to osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, peridontal disease, tumoral calcinosis and dermatomyotsitis.

ERRα can be used both *in vitro* and *in vivo* to stimulate proliferation and differentiation of osteoblasts from primitive progenitors to mature osteoblasts and osteocytes. Thus it has therapeutic utility for increasing bone mass in such conditions where bone mass is decreased as for example in osteoporosis.

ERRα mRNA is differentially expressed in developing osteoblast lineage cells

It is now demonstrated that ERRα mRNA is expressed in differentiating primary cultures of rat calvaria cells and in single isolated osteoblast colonies at different stages of differentiation. However, levels were generally lower in primitive progenitors, more mature precursors, and osteoblastic cells associated with mineralized nodules, and higher in preosteoblasts and osteoblasts. These results suggest that ERRα may have a function in osteoblasts throughout their developmental lifetime, ie. from primitive progenitor to the mature osteoblasts and osteocytes, and throughout the lifetime of the organism, i.e., ERRα protein was detected *in vivo* in 21 day fetal (see also below) and in adult calvaria, where ERRα is still expressed in osteocytes (data not shown). It has also been demonstrated that ERRα is expressed throughout osteogenesis in rat bone marrow stromal cell cultures (data not shown). These results suggest that ERRα may have functions not only during fetal development but also in adult life in both bone and bone marrow stroma. Strikingly, ERRα mRNA is also more highly expressed that either ERα or ERβ mRNA. Taken together, these data may indicate that ERRα has a more widespread activity in bone than either ERα and ERβ.

ERRα mRNA is more highly and widely expressed than either ERα and ERβ

ERRα protein was found more widely distributed *in vitro* in RC cell cultures than either ERα or ERβ. ERRα was found in most if not all cells in RC cell cultures from early proliferation stages through mineralized nodule formation. ERα was also detected in RC cells at all times analysed but at lower levels than ERRα which is also the case in rat bone marrow cultures (data not shown). ERβ, on the other hand, was more difficult to detect at any time other than in early proliferating cultures. These observations fit with the expression pattern of these three receptors *in vivo* in 21 day fetal calvariae. Indeed, ERRα is more highly and widely expressed than either ERα or ERβ, being highly expressed in sutural cells and all identifiable osteoblasts and osteocytes. ERα is not highly expressed in nascent, but is detectable in more mature, osteoblastic cells. ERβ, on the other hand, is more highly expressed in sutural and nascent osteoblasts. These results suggest that ERRα and ERα may be co-expressed in at least some osteoblastic cells, and that these receptors may regulate the expression of the same target genes in bone via their known ability to participate in protein-protein interactions (Johnston et al, 1997) and their recently described capacity to bind to the same DNA target sequence on the osteopontin promoter (Vanacker et al, 1999). Whether ERRα and ERβ co-expression also occurs in early osteoblastic cells (osteoprogenitors) is a possibility based on the present data but interactions between these two receptors has not yet been described although they do recognize the same ERE response element as recently described (Vanacker et al, 1999). It is also notable that ERRα protein is localized primarily in either the nucleus or the cytoplasm or both depending on the developmental stage of the osteoblast. This suggests that ERRα target genes and funciton may vary depending on the depending on the maturational stage of the osteoblastic cells.

ERRα is required for proliferation of RC cells and for the formation of bone nodules

Consistent with its expression in proliferating RC cells, we have found that downregulation of ERRα inhibits proliferation of RC cell populations, an inhibition that may have consequences on bone nodule formation at later times. However, downregulation of ERRα also decreases differentiation and bone nodule formation in a proliferation-independent manner in RC cells cultures. Upregulation of ERRα had biphasic effects on bone formation, either increasing or decreasing bone nodule formation depending on the amount of overexpressed ERRα. These results suggest an essential role for ERRα in proliferation, differentiation and bone nodule formation in osteoblastic populations *in vitro*, predicting an important role for this receptor in the formation and turnover of the skeleton. Bone specific knockout of ERRα or ERRα overexpression experiments in transgenic mice will test these possibilities.

These functions of ERRα correlate with the known function of ERα in bone. For example, the knockout of ERα caused a 20% reduction in bone mass (Korach, 1994). The fact that ERRα and ERα appear to be co-expressed in at least some osteoblast populations, and may have the capacity to functionally interact, suggests that they may have at least some of the same functions in bone, including an activity in bone formation.

ERRα expression is stimulated by estrogen and vitamin D3 but not by dexamethasone in proliferating RC cell cultures

ERRα is expressed in RC cell populations and the level of expression is almost the same in cells not treated (-Dex) or treated with dexamethasone (+ Dex). A 24 hour acute treatment of RC cells with Dex at day 9 or day 15 also had no effect on the ERRα expression level. These results suggest that Dex has no effect on the expression of ERRα while it does downregulate ERα and even more potently downregulates ERβ. These results suggest that the regulation by glucocorticoids of ERRα differs from that of the ERs.

It was also demonstrated that estrogen (E2) regulates ERRα at early times in chronically treated RC cell cultures, while an acute (24h) treatment at either day 9 or day 15 did not. These results suggest a link between ERRα and E2 in bone during proliferation. Shigeta et al. showed that E2 can also activate ERRα in the uterus (Shigeta et al, 1997). Together, these results suggest a link between ERRα and estrogens in two estrogen-sensitive tissues.

ERRα and ERα are both expressed in adult osteocytes suggesting a function of ERRα during adult life and, consequently, may be involved in some diseases characterized by a decrease in bone mass such as osteoporosis. Bone loss in the aging skeleton is accelerated by a decrease in secretion of estrogens in post-menopausal women and can be reversed by administration of natural or synthetic estrogens. The decrease in estrogen also induces a decrease in the expression of ERα (Braidman et al, 1998). It is possible that ERRα is also regulated by estrogens in the aging skeleton and may have a role in osteoporosis.

ERRα is also regulated by another hormone involved in bone metabolism, namely 1,25 $(OH)_2$ D_3, during the proliferation stages of RC cell cultures. As with E2, acute treatment with 1,25 $(OH)_2$ D_3 for 24 hours at day 9 or day 15 had no effect on ERRα expression. It will be determined whether $1,25(OH)_2D_3$ effects may be mediated at least in part by its abilities to modify ERRα levels.

In conclusion, ERRα is expressed in osteoblastic cells at levels higher than either ERα or ERβ. ERRα appears to have a function in proliferation, differentiation and bone nodules formation in RC cells. Finally, ERRα is regulated by E2 and 1,25 $(OH)_2$ D_3, two important hormones known to have strong effects on bone formation and turnover. All these results appear to converge in the same pathway and suggest that ERRα may have a important function in the formation and turnover of the skeleton.

Transgenic non-human animal models may be made for the study of the effects of over and under expression of the ERRα gene, for the screening of candidate compounds as potential antagonists of this receptor and for the evaluation of potential therapeutic interventions.

The transgenic animals of the invention may also provide models of disease conditions associated with abnormalities of ERRα expression. For example, the transgenic animals of the invention may provide an animal model of at least some aspects of rheumatoid arthritis

Animal species suitable for use in the animal models of the invention include mice, rats, rabbits, dogs, cats, goats, sheep, pigs and non-human primates.

Animal models may be produced by inserting a selected nucleic acid sequence into a germ line cell or a stem cell using previously described techniques such as oocyte microinjection or transfection or microinjection into embryonic stem cells. Alternatively, an endogenous ERRα gene may be inactivated or replaced by homologous recombination within embryonic stem cells to produce "knock-out" or "knock-in" animal models. Techniques for obtaining transgenic animals are widely available in the literature.

In accordance with one embodiment of the invention, transgenic animals generated by the introduction of a ERRα transgene into a fertilized animal oocyte, with subsequent growth of the embryo to birth as a live animal. The ERRα transgene is a transcription unit which directs the expression of ERRα gene in eukaryotic cells. To create the transgene, ERRα gene is ligated with an eukaryotic expression module. The basic eukaryotic expression module contains a promoter element to mediate transcription of ERRα sequences and signals required for efficient for termination and polyadenylation of the transcript. Additional elements of the module may include enhancers which stimulate transcription of ERRα sequences. The most frequently utilized termination and polyadenylation signals are those derived from SV40 (5) The choice of promoter and enhancer elements to be incorporated into the ERRα transgene is determined by the cell types in which ERRα gene is to be expressed. To achieve expression in a broad range of cells, promoter and enhancer elements derived from viruses may be utilized, such as the herpes simplex virus thymidine kinase promoter and polyoma enhancer. To achieve

exclusive expressio[redacted] particular cell type, specific promoter an[redacted]hancer elements could be used, such as the promoter of the mb-1 gene and the intronic enhancer of the immunoglobulin heavy chain gene. In the present invention, it would be preferred to achieve expression in osteoblasts for example and use a bone specific promoter of bone sialoprotein

The ERRα transgene is inserted into a plasmid vector, such as pBR322 for amplification. The entire ERRα transgene is then released from the plasmid by enzyme digestion, purified and injected into an oocyte. The oocyte is subsequently implanted into a pseudopregnant female animal. Southern blot analysis or other approaches are used to determined the genotype of the founder animals and animals generated in the subsequent backcross and intercross

Transgenic mice deficient in the production of ERRα may also be made by homologous recombination. Methods of disrupting the genes of an animal are well established and are described in various publications.

Such deficient mice will provide a model for study of the role of ERRα in bone cell differentiation and proliferation and general skeletal development. Such animals will also provide tools for screening candidate compounds for their interaction with ERRα or the signalling pathway activated by ERRα.

In a further embodiment, the invention provides pharmaceutical compositions comprising ERRα or a functional analogue or mimetic of ERRα for the treatment of certain skeletal and connective tissue disorders in which ERRα expression is abnormal. Such disorders may include but are not limited to osteoporosis, rheumatoid arthritis, osteoarthritis, fibrodysplasia ossificans progressiva, Paget's disease, peridontal disease, tumoral calcinosis and dermatomyotsitis. Such compositions as provided herein can be appropriately packaged and targeted to specific cells and/or tissues.

Administration of a therapeutically active amount of a pharmaceutical composition of the present invention means an amount effective, at dosages and for periods of time necessary to achieve the desired result. This may also vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the ERRα to elicit a desired response in the subject. Dosage regima may be adjusted to provide the optimum

therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation

By pharmaceutically acceptable carrier as used herein is meant one or more compatible solid or liquid delivery systems. Some examples of pharmaceutically acceptable carriers are sugars, starches, cellulose and its derivatives, powdered tragacanth, malt, gelatin, collagen, talc, stearic acids, magnesium stearate, calcium sulfate, vegetable oils, polyols, agar, alginic acids, pyrogen-free water, isotonic saline, phosphate buffer, and other suitable non-toxic substances used in pharmaceutical formulations. Other excipients such as wetting agents and lubricants, tableting agents, stabilizers, anti-oxidants and preservatives are also contemplated

The compositions described herein can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable carrier Suitable carriers and formulations adapted for particular modes of administration are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis the compositions include, albeit not exclusively, solutions of the substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions of the invention may be administered therapeutically by various routes such as by injection or by oral, nasal, buccal, rectal, vaginal, transdermal or ocular routes in a variety of formulations, as is known to those skilled in the art.

The present invention also enables the analysis of factors affecting the expression of the ERRα gene in humans or in animal models. The invention further provides a system for screening candidate compounds for their ability to turn on or turn off expression of the ERRα gene and the identification of binding partners which may also affect expression of ERRα or certain downstream partners.

For example [redacted]C cell culture system can be used to ide[redacted] compounds which activate production of ERRα or, once ERRα production has been activated in the cells, they can be used to identify compounds which lead to suppression or switching off of ERRα production. Alternatively, such a cell culture system can be used to identify compounds or binding partners of ERRα which increase its expression. Compounds thus identified are useful as therapeutics in conditions where ERRα production is deficient or excessive.

The present invention enables also a screening method for compounds of therapeutic utility as antagonists of the biological activity of ERRα. Such antagonist compounds are useful, for example, to reduce or prevent differentiation and maturation of osteoblasts and osteocytes. ERRα antagonists may also be used in the treatment of bone related disorders involved inappropriate bone cell growth. Those skilled in the art will be able to devise a number of possible screening methods for screening candidate compounds for ERRα antagonism.

A screening method may also be based on binding to the ERRα receptor. Such competitive binding assays are well known to those skilled in the art. Once binding has been established for a particular compound, a biological activity assay is employed to determine agonist or antagonist potential.

The present invention further enables therapeutic intervention in disorders associated with an inappropriate level or location of ERRα expression.

Such interventions include;

(a) in conditions associated with undesired biological activity of ERRα, inhibition of its activity by administration of antagonist compounds, anti- ERRα antibodies, agents to down-regulate gene expression or alternatively antisense methods to inhibit gene function. ERRα can be conjugated with selected molecules to target specific tissues inappropriately over producing ERRα.

Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of chemistry, molecular biology, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

ERRα is differentially expressed as osteoprogenitor cells differentiate to mature osteoblasts and osteocytes

ERRα mRNA expression levels were assessed over a proliferation-differentiation time course by Northern blotting of primary rat calvaria (RC) cells grown in the presence (+Dex) or absence (-Dex) of dexamethasone (Dex). In cultures grown without Dex, in which few nodules formed, ERRα mRNA was expressed in proliferating RC cells (day 6), but decreased to lower levels in cells undergoing condensation and early nodule formation (day 10) and nodule mineralization (day 15) (Fig. 1). In cultures treated with Dex, in which nodule formation was increased significantly, ERRα levels were similar to those seen in -Dex cultures at all time points (Fig. 1). For comparison, mRNA levels for two osteoblast markers, alkaline phosphatase, a relatively early marker of osteoblast development, and osteocalcin, a marker of osteoblast maturation, are also shown.

Because RC cell cultures comprise a heterogeneous mixture of cell types and osteoblasts at different differentiation stages, it was sought to confirm that ERRα is expressed by osteoblast lineage cells. Globally-amplified (poly(A) PCR) cDNA pools prepared previously from single isolated osteoblast colonies at different stages of differentiation were used to do this. Colonies used were selected based on their molecular phenotypes (expression of osteopontin, bone sialoprotein, alkaline phosphatase and osteocalcin). ERRα was amplified in each cDNA pool with specific primers for sequences in the 3' UTR of ERRα. ERRα was found to be expressed at all developmental times

(Fig 2) Notably, [redacted] levels were generally lower in primitive progenitors (A), more mature precursors (B), and osteoblastic cells associated with mineralized nodules (E), and higher in preosteoblasts (C) and osteoblasts (D) (Fig 2)

ERRα mRNA is more highly expressed in RC cell cultures than either ERα and ERβ mRNA and expression patterns vary

The level of ERRα expression was compared with those of the two estrogen receptors, ERα and ERβ. RT-PCR was done with primers specific for each of these three receptors. ERRα was found to be expressed at significantly higher levels than either ERα and ERβ and the two estrogen receptors were themselves present at different levels (i.e., ERRα was easily detected at 25 cycles, while 35 cycles and 40 cycles were required to detect ERα and ERβ respectively). In addition, the expression patterns of the three receptors over the proliferation-differentiation time course in RC cell cultures was strikingly different. Similarly to ERRα, ERβ decreased over time in -Dex cultures, whereas ERα increased (Fig 3). On the other hand, both ERα and ERβ decreased over time in +Dex cultures in a manner similar to ERRα (cf. Fig 1 and 3).

Cell culture

For cell cultures, cells were enzymatically isolated from the calvaria of 21 d Wistar rat fetuses by sequential digestion with collagenase as described previously (Bellows, 1986). Cells obtained from the last four of the five digestion steps (populations II-V) were pooled and plated in T-75 flasks in α-MEM containing 15% heat-inactivated FBS (Flow Laboratories, McLean, VA) and antibiotics comprising 100 mg/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 mg/ml gentamycin (Sigma), and 0.3 mg/ml fungizone (Flow Laboratories). After 24hrs incubation, attached cells were washed with PBS to remove nonviable cells and other debris, and then collected by trypsinization using 0.01% trypsin in citrate saline. Aliquots were counted with a Coulter Counter, and the remaining

cells were resuspended in the standard medium described above The resuspended cells were plated into 100 mm tissue culture dishes at 10^5 cells, into 35mm tissue culture dishes at 2 x 10^4 and in 24 wells plates at 10^4 After 24hrs incubation, medium was changed and supplemented with 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and with or without 10^{-8} M dexamethasone, 10^{-8} M of E2 (Merck, Sharp, and Dohme, Canada, Ltd., Kirkland, PQ) or 10^{-8} M of E2 (Sigma), or 10^{-9} M of 1,25Vit D3. Medium was changed every 2 days. All dishes were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 incubator.

Northern blots

Total RNA was extracted with guanidine from RC cells at different times of the culture corresponding to different stages of proliferation, differentiation and bone nodule formation (Current Protocols in Molecular Biology, vol. 1, 1996). Northern blots were prepared and hybridized with a 750bp fragment corresponding to the rat 3' UTR of ERRα (J.M. Vanacker) according to standard procedures (Chirgwin et al, 1979). Rat bone/liver/kidney ALP (Noda et al., 1987; gift of Dr. G.A. Rodan, Merck Sharpe, and Dohme Research Laboratories, West Point, PA) was a 600 bp cDNA *EcoRI* fragment obtained by digesting pRAP54 with *BssHII-XhoI* to remove 1.8 kb of 5' region and religating the blunt ends. Rat OPN (Dr R. Mukherjee, Montreal, PQ) was a 700 bp cDNA *BamHI-EcoRI* fragment obtained by digesting full length cDNA with *PvuI* to remove 800 bp of 5' region and ligating the blunt ended fragment into *SmaI* cut pGEM-7Zf(+) vector (Promega, Madison, WI). Rat OCN was a partial cDNA containing 350 bp of the 3' UTR isolated with OCN-specific primers from a λgt11 library prepared from ROS 17/2.8 cells. Identity was confirmed by sequencing (Liu et al., 1994).

RT-PCR

Samples of total cellular RNA (5μg) were reverse-transcribed using oligo dT and the first strand synthesis kit of Superscript™ II. Gibco BRL. PCR was performed with primers specific for ERRα, ERα and ERβ. Primers, located in different exons, were as follows

ERRα upstream (3'UTR)	CAG GAA AGT GAA TGC CCA GG
ERRα downstream (3'UTR)	CTT TGC AGC AAA TAT ACA TT
ERα upstream (Dom D 5')	GAG CTG CCA ACC TTT GGC CAA GT
ERα downstream (Dom D 3')	TGA ACT TGA TCG TGG AGA TTC
ERβ upstream (Dom D)	AAA GCC AAG AGA AAC GGT GGG CAT
ERβ downsteam (Dom E)	GCC AAT CAT GTG CAC CAG TTC CTT
L32 upstream	CAT GGC TGC CCT TCG GCC TC
L32 downstream	CAT TCT CTT CGC TGC GTA GCC

The PCR reaction mixture contained cDNA (1μl), 1μl dNTP mix (20mM), 10x PCR buffer, 25pmol primers and 5 Units of Taq polymerase from Quiagen. PCR was done for 25 cycles (94°C for 1 min, 55°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ERRα and L32; 35 cycles (94°C for 1 min, 55°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ERα, 45 cycles (94°C for 1 min, 59°C for 1min, 72°C for 1min and a final elongation step of 7 min at 72°C) for ERβ. Amplimers were sequenced for verification.

Poly (A) PCR Library selection

Twenty poly(A) PCR libraries representative of five transitional stages in osteoblast lineage progression were selected from more than one hundred available amplified colonies (Liu,F and Aubin, J.E., 1998) Stage A are replica-plated monolayer colonies commited to differentiate to the osteoblast lineage but not yet expressing type I

αI collagen or alkaline phosphatase, both early markers of osteoprogenitor cells. Stage B and C colonies are progressively more mature, i e expressing type I αI collagen or both type I αI collagen and alkaline phosphatase respectively. Stage D colonies represent multilayered cells and contain histologically identifiable cuboidal osteoblasts. Stage E colonies comprised terminal differentiation stages, with multilayered cells and mineralized bone matrix. Relative amounts of total cDNA were determined by Southern hybridization and were used for normalization

Western blots

Total protein was extracted from confluent HeLa cells according to standard methods (Current Protocols in Molecular Biology, vol. 1, 1996) and Western blot analyses were performed using a semi-dry system. Immunoblotting was performed with rabbit antiserum prepared against a rat peptide (NH-CPASDECEITKRR-C) localized in the C domain of ERRα (J.M. Vanacker). Blots were incubated overnight at room temperature with the antiserum diluted to 1/500, and binding was detected using HRPO -conjugated goat-anti-rabbit antibodies (Biorad).

ERRα, ERα and ERβ protein is also expressed in RC cultures, but only ERRα and ERα are detectable in bone nodules

To determine whether and when in RC cultures the three receptors were expressed, immunocytochemistry was performed using polyclonal antibodies specific for ERα and ERβ (Santa Cruz) and antiserum prepared against ERRα in +Dex-treated RC cultures. To confirm the specificity of the ERRα antiserum, a Western blot on Hela cells was done. As expected based on previously published data (Johnston et al, 1997, Shigeta et al, 1997), a single immunoreactive band at 53Kd in Hela cells (Fig 4R) was detected. Interestingly ERα was detected in RC cells at all times analysed from early proliferation stages through nodule formation and mineralization (Fig. 4 B, E, H, K, N, Q); note

especially the strong labeling of osteoblastic cells in nodules (Fig. 4N, Q). ERβ, on the other hand, was more difficult to detect at any time other than in early proliferating cultures (Fig. 4C, F, I, L, O), in particular, ERβ in osteoblastic cells in bone nodules (Fig 4O) was seldom detected.

As predicted based on the relatively wide tissue distribution of ERRα mRNA (Bonnelye et al., 1997b), ERRα protein was found more widely distributed in RC cell cultures than either ERα or ERβ. ERRα was found in most if not all cells in RC cell cultures from early proliferation stages through mineralized nodule formation (Fig. 4A, D, G, J, M, P); note especially, however, that staining for ERRα was more intense in the osteoblasts associated with both early and late bone nodules than in the surrounding fibroblastic cells (Fig. 4J, M). Interestingly, however, while ERRα is primarily found in the cytoplasm and perinuclear location at day 2 and day 4 (Fig. 4 A, D), by day 6 and thereafter, including in mature osteoblasts, it is primarily localized in the nucleus (Fig. 4G, J, M, P). ERα was mainly nuclear from day 2 (Fig. 4B) to day 6 but thereafter was cytoplasmic and nuclear (Fig. 4 N, Q), while ERβ was primarily perinuclear in all cells in which it could be detected (e.g., Fig. 4F).

ERRα is more highly and widely expressed *in vivo* in fetal rat calvaria compared ERα and ERβ

To extend the observations made *in vitro* to bones *in vivo*, immunocytochemistry was performed on 21d. fetal rat calvaria sections. Consistent with the *in vitro* results, strikingly different expression patterns were seen for ERRα, ERα and ERβ. ERRα was found in all detectable cohorts of osteoblasts from those associated with nascent bone at the osteogenic front (Fig 5A) to those in more mature bone trabeculae (Fig 5B) including spongy bone (Fig 5C). Consistent with our RT-PCR results on single bone nodules (Fig. 2), ERRα was also detectable in sutural cells (Fig. 5A), preosteoblasts (Fig. 5C) and osteocytes (Fig. 5B). ERα, on the other hand, was not detected in any cells in the suture

or osteogenic front (Fig. 5D), but was detected in some osteoblasts associated with more mature (Fig. 5E) and spongy bone (Fig 5F) ERβ was detected in a pattern virtually reciprocal to that of ERα, i e , it was present in sutural cells and cells at the osteogenic front (Fig 5G), but it was virtually undetectable in osteoblastic cells in more mature and spongy bone (Fig. 5H, I). Based on staining intensity, and in keeping with our RT-PCR results, ERRα was more highly expressed than either ERα and ERβ *in vivo*

Immunolabelling

Immunolabelling of cultures was done essentially as described previously (Turksen, 1991 , Turksen, 1992). Cultures were rinsed with PBS, fixed with 3 7% formaldehyde in PBS and permeabilized with methanol at -20°C Frozen sections were fixed 10 min in cold acetone. After rinsing, cells in dishes or frozen sections were incubated for 1 hr at room temperature with 10% normal serum in PBS for ERRα and ERα and in 3% BSA in PBS (denaturated) for ERβ. After rinsing, cells or sections were incubated for 1.5 hours with appropriate dilutions of primary antibodies (1/500, anti-ERRα, as above; anti-ERα or anti- Erβ (MC-20 or Y-19, respectively, Santa Cruz Biotechnology, Inc)). 10% normal serum in PBS or 3% BSA in PBS were used as negative controls. Nodules or calvaria sections were rinsed in PBS and incubated for 1hr at room temperature with CY-3-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Lab, West Grove, PA, USA; 1/300 final dilution) for ERRα and ERα and with an anti-goat antibody for ERβ. After rinsing, samples were mounted in Moviol (Hoechst Ltd, Montreal, PQ, Canada) and observed by epifluorescence microscopy on a Zeiss Photomicroscope III (Zeiss, Oberkochen, Germany). For photography and printing, equal exposure times were used for specifically-labelled and control cultures.

In Situ Hybridizati

Frozen sections for *in situ* hybridization were fixed for 20 minutes in 4% paraformalde[illegible] dehydrated and stored until used at -70°C. The hybridization protocol was as described previously (Zellar and Rogers, 1989), except that sections were not treated with pronase so as to preserve the cellular morphology. Briefly, sections were acetylated and RNA probes in hybridization mix (0.48M NaCl, 8mM Tris-Cl,pH 7.5, 1.6 mM EDTA, 0.8x Denhardt's solution, 0.4 mg/ml yeast tRNA, 80mg/ml poly(A), 40% deionized formamide, 20% dextran sulfate) were applied for hybridization for 4 hours at 42°C. After hybridization, sections were washed at 50°C twice with wash solution I (2x SSC, 50% Formamide, 0.1% β-mercaptoethanol), treated with Rnase A (20mg/ml boiled Rnase A, 0.5 M NaCl, 10mM Tris-Cl) for 1hour at 37°C and washed with wash solution II (0.1x SSC, 1% β-mercaptoethanol) as descrided (Zellar and Rogers, 1989). The ^{35}UTP-labelled RNA probe used was 750bp fragment of the 3'UTR of rat ERRα.

Nodule quantification

For quantification of nodule formation, dishes or wells were fixed and stained by the Von Kossa technique and bone nodules were counted on a grid (Bellows et al., 1986, Bellows and Aubin, 1989). Results are plotted as the mean number of nodules ± SD of three dishes for controls and each concentration of antisense or sense primers.

Cell counting

For cell growth analysis, the cell layers were rinsed in PBS, released with trypsin and collagenase (1:1, vol/vol, of solutions described above), and the harvested cells were counted electronically. Results are plotted as the averge of three counts for each of two dishes for each concentration of antisense or sense primers used

Alkaline phosphatase histochemistry

The histochemical stain for alkaline phosphatase is a modification of Pearse's (1960). Cells were rinsed once with cold PBS and fixed in 10% cold neutral buffered formalin for 15 min, rinsed with distilled water, and left in distilled water for 15 min. Fresh substrate (10 mg Naphthol AS MX-PO4 (Sigma) dissolved in 400 µl N,N-dimethylformamide, then added to 50 ml distilled water and 50 ml Tris-HCl (0.2 M, pH 8.3) and then 60 mg Red Violet LB salt (Sigma)), was filtered through Whatman's No. 1 filter directly onto the dishes, and incubated for 45 min at 20°C. The dishes were then rinsed in tap water, drained and stained with 2.5% silver nitrate for 30 min at room temperature (von Kossa stain). After rinsing twice in distilled water, toluidine blue was applied for 2 seconds and the dishes were then rinsed 3 times with tap water. The dishes were finally air dried.

Transient transfections

Primary RC cells were grown in 35mm tissue culture dishes at 2 x 10^4/dish in α-MEM containing 10% heat-inactivated FBS (Flow Laboratories, McLean, VA) and supplemented with 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and 10^{-8} M dexamethasone. Cells were transfected at 50% of confluence according to the Effecten transfection protocol (Quiagen) using a pcDNA empty plasmid as a control and pcDNA3-ERRα (in the EcoRI cloning site) at 0.5 to 1µg of total DNA per transfection. As control of transfection efficiency, a CMV- βGal vector was used. Nodules were counted at 15 days.

ERRα is required for proliferation of RC cells and for the formation of bone nodules

Given its expression in both proliferating osteoprogenitor cells and more mature osteoblasts and osteocytes, it was next determined whether ERRα is a critical factor in osteoblast proliferation or differentiation. Antisense oligonucleotides form DNA:RNA

duplexes with specific mRNA species, thereby blocking binding of the mRNA to the 40S ribosomal subunit and preventing translation (Reddy et al. 1994). An antisense oligonucleotide specific for ERRα was used and found a dose dependent decrease in cell number at day 2 (Fig 6). These results suggest that ERRα plays a role in the proliferation of RC cells, an effect that may also influence bone nodule number later in treated cultures.

To determine whether ERRα may play a role in osteoblast differentiation, independently of an effect on proliferation, RC cells were treated with the antisense oligonucleotide beginning at day 6 (after cells had reached confluence). While sense oligonucleotides had a small dose dependent effect on nodule numbers, antisense oligonucleotides caused a striking dose dependent decrease in bone nodule formation, i.e., 70% at 0.5μM, and 100% at 1 μM and 2μM (Fig 7). On the other hand, overexpression of ERRα with transient transfection of RC cells, resulted in a dose dependent biphasic effect. transfection with 0.5μg of ERRα-containing vector increased (~ 20%) the number of mineralized bone nodules whereas transfection with 0.5μg of ERRα-containing vector decreased (40%) the number (Fig 8)

ERRα expression is stimulated by estrogen and vitamin D3 in proliferating RC cells cultures

To determine whether other hormones that influence proliferation and differentiation in RC cell cultures may modulate ERRα levels, RC cells were treated continuously with estrogen (E2, 10^{-8}M) or 1,25$(OH)_2$ vitamin D_3 (D3, 10^{-9}M). Both E2 (40% increase) and D3 (47% increase) stimulated expression of ERRα mRNA at day 6, but not later (Fig 9). An acute exposure of RC cells to either E2 or D3 for 24 hours at beginning from day 9 (nascent nodule formation) or day 15 (mature nodules present) had any effect on ERRα mRNA levels (Fig 9)

Antisense and sense oligonucleotide treatment

The resuspended cells were plated /in 24 wells plates at 10^4/dish. Antisense oligonucleotide inhibition of ERRα expression was accomplished with a 20-base phosphorothioate-modified oligonucleotide, localized to the A/B domain. The ERRα antisense oligonucleotide sequence was 5'-TCACCGGGGGTTCAGTCTCA-3'. Control dishes were treated with the complementary sense oligonucleotide or no oligonucleotide. Preliminary experiments were done to determine effective oligonucleotide concentrations that were not toxic. 0.1μM to 5μM oligonucleotides were added directly to cells either during the proliferation phase (days 1 to 6) and 0.1μM to 2μM oligonucleotides were added for the differentiation phase (days 6 to 12) in standard medium as above supplemented with 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and 10^{-8} M dexamethasone. Medium was changed every 2 days and fresh oligonucleotides were added.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is decribed.

References

Bellow.C.G , and Aubin,J E (1989) *Dev. Biol* 133,8-13

Bellow.C.G , Aubin,J E , Heersche, J N M and Antosz, M.E (1986) *Calcified Tissue Int* ,38 143-154

Bonnelye E , JM Vanacker, X Desbiens, T Dittmer, A.Begue, J.Aubin, V Laudet and B.Fournier *Mol Endocrinol*, 11, 1997 905-916 (a)

Bonnelye E, JM Vanacker, S Alric, N.Spruyt, B Fournier, X.Desbien and V Laudet *MOD*, 65, 1997 71-85 (b)

Braidman, I , Baris, C , Wood, L , Selby,P , Freemont, A., Hoyland, J , (1998) *J, Bone and Mineral Research*, vol 23 5 S201

Chirgwin,J.J , Przbyla,A.E , MacDonald, R.J., and Rutter,W J (1979) In *Current CP protocols* pp 4 2 1-4,2 8 John Wiley&Sons, Inc

Denhardt,D.T and Guo,X (1993) *FASEB* J ,7,1475-1482

Enmark,E and Gustafsson,J-A (1996) *Mol.Endocrinol* ,10,1293-1307

Giguère V, Yang N, Segui P, Evans RM 1988 *Nature* 331 : 91-94

Green,S , Walter,P , Kumar,V, Krust.A , Bornert,J M , Argos,P and Chambon,P (1986) *Nature*, 320,134-139

Gronemeyer H, Laudet V 1995 Transcription factors 3: nuclear receptors. Protein profile 2 : 1173-1308

Johnston,S.D , X.Liu, F Zuo, T.L.Eisenbraun, S.R.Wiley, R.J Kraus and J.E.Mertz. *Mol.Endocrinol* (1997) 11 : 342-352.

Korach,K.S (1994) *Science*, 266, 1524-1527

Kuiper,G.G JM, Enmark,E , Pelto-Huikko,M., Nilsson,S. and Gustafsson,J.A (1996) *Proc.Natl Acad. Sci. USA*, 93, 5925-5930

Liu,F , Malaval L., Gupta,A K , and Aubin,J.E.(1994) *Dev.Biol* 166,220-234.

Liu,F & Aubin,J.E (1998) *Dev. Biol* (for review)

Noda,M , Yoon,K ,Thiede,M., Buenaga,R., Weiss,M., Henthorn,P., Harris,H., and Rodan,G. (1987) *J.Bone Miner. Res.*2,161-164.

Pacifici, R (1996) *J.Bone Miner.Res.*, 11, 1043-1051

Pettersson K, Svensson K, Mattsson R, Carlsson B, Ohlsson R, Berkenstam A 1996 *Mech. Dev.* 54 : 211-213

Shigeta, H., Zuo, W , DiAugustine, R., and Teng C,T (1997) *J. of Molecular Endocrinology.* 19, 299-309

Sladek,R., J.A Bader and V.Giguère. *Mol.Cell.Biol.* (1997) 17 : 5400-5409

Turksen, K., and Aubin,J.E. (1991) *J.Cell Biol.* 114, 373-384

Turksen,K., Bhargava, U., Moe,H K., and Aubin, J.E. (1992) *J. Histochem. Cytochem.*40,1339-1352

Turner RT, Riggs BL, Spelsberg TC. 1994. *Endocr. Rev.* 15 : 275-300

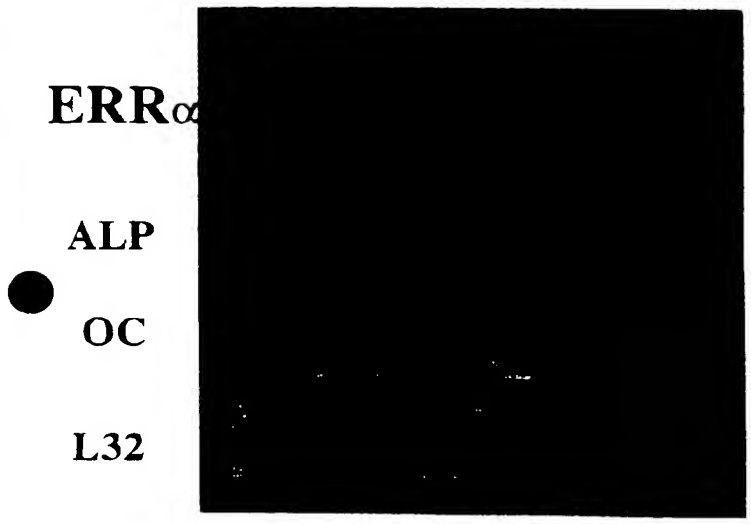
Vanacker,J-M., Pettersson, K., Gustafsson, J-A., and Laudet, V. (1999). *EMBO* 18, 4270-4279.

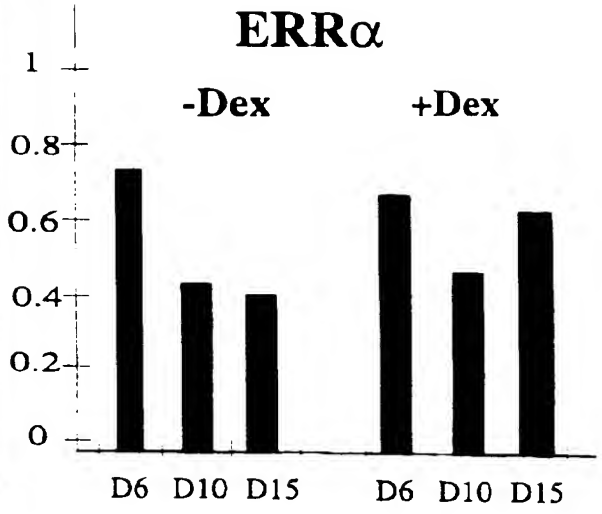
Vanacker,J.-M., Delmarre,C., Guo,X. and Laudet,V. (1998a) *Cell Growth Differ.*, 9,1007-1014.

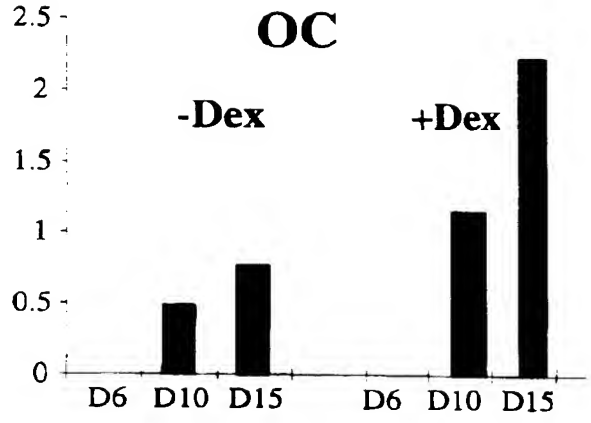
Wiley SR, Kraus RJ, Zuo F, Murray EE, Loritz K, Mertz JE 1993 *Genes and Dev.* 7 : 2206-2219

Yang N, Shigeta H, Shi H, Teng CT 1996 *J. Biol. Chem.* 271 : 5795-5804

Zellar,R. and Rogers, M (1989) In *Current CP protocols* , pp.14.3.1-14.3.14. John Wiley&Sons, Inc


ERRα
ALP
OC
L32


ERRα
-Dex
+Dex
1
0.8
0.6
0.4
0.2
0
D6 D10 D15
D6 D10 D15


OC
-Dex
+Dex
2.5
2
1.5
1
0.5
0
D6 D10 D15
D6 D10 D15

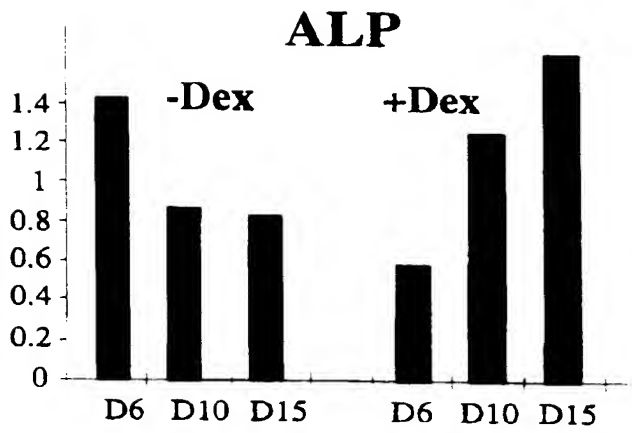

ALP
-Dex
+Dex
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D6 D10 D15
D6 D10 D15

Figure 1

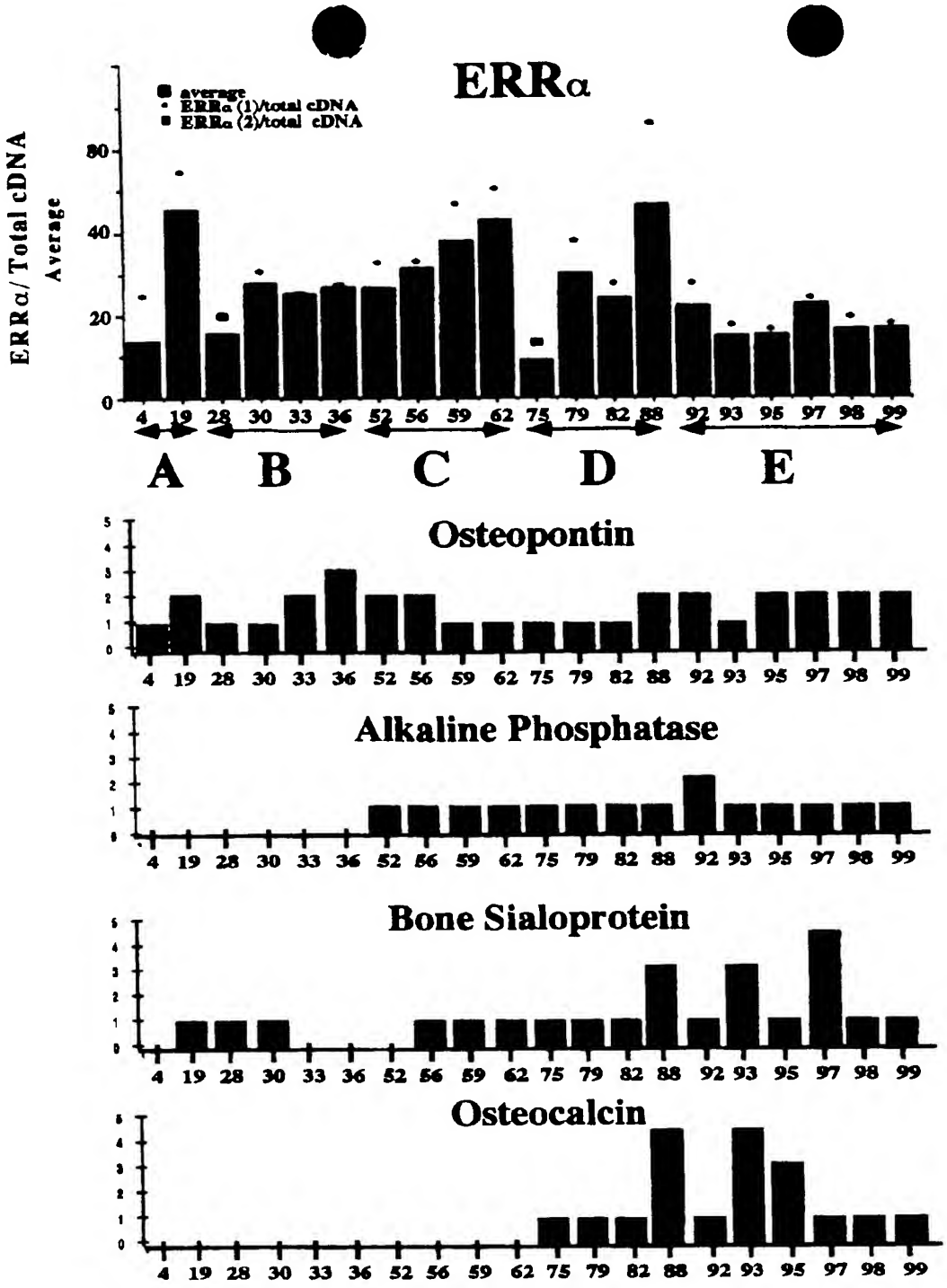

ERRα
average
ERRα (1)/total cDNA
ERRα (2)/total cDNA
ERRα/ Total cDNA
Average
0
20
40
80
4 19 28 30 33 36 52 56 59 62 75 79 82 88 92 93 95 97 98 99
A
B
C
D
E
Osteopontin
Alkaline Phosphatase
Bone Sialoprotein
Osteocalcin

Figure 2

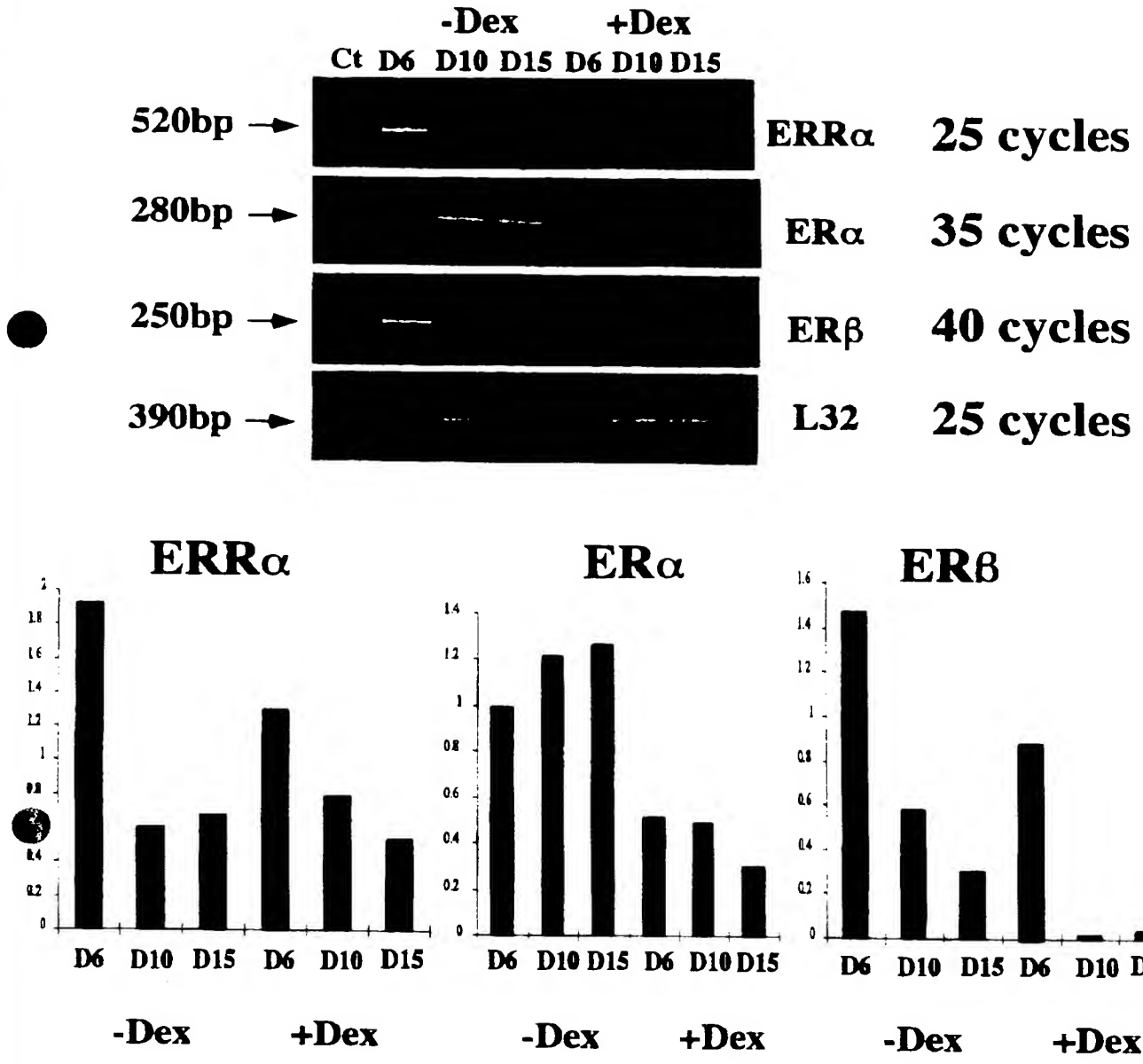

-Dex
+Dex
Ct D6 D10 D15 D6 D10 D15
520bp
ERRα
25 cycles
280bp
ERα
35 cycles
250bp
ERβ
40 cycles
390bp
L32
25 cycles
ERRα
ERα
ERβ
D6 D10 D15 D6 D10 D15
-Dex
+Dex

Figure 3

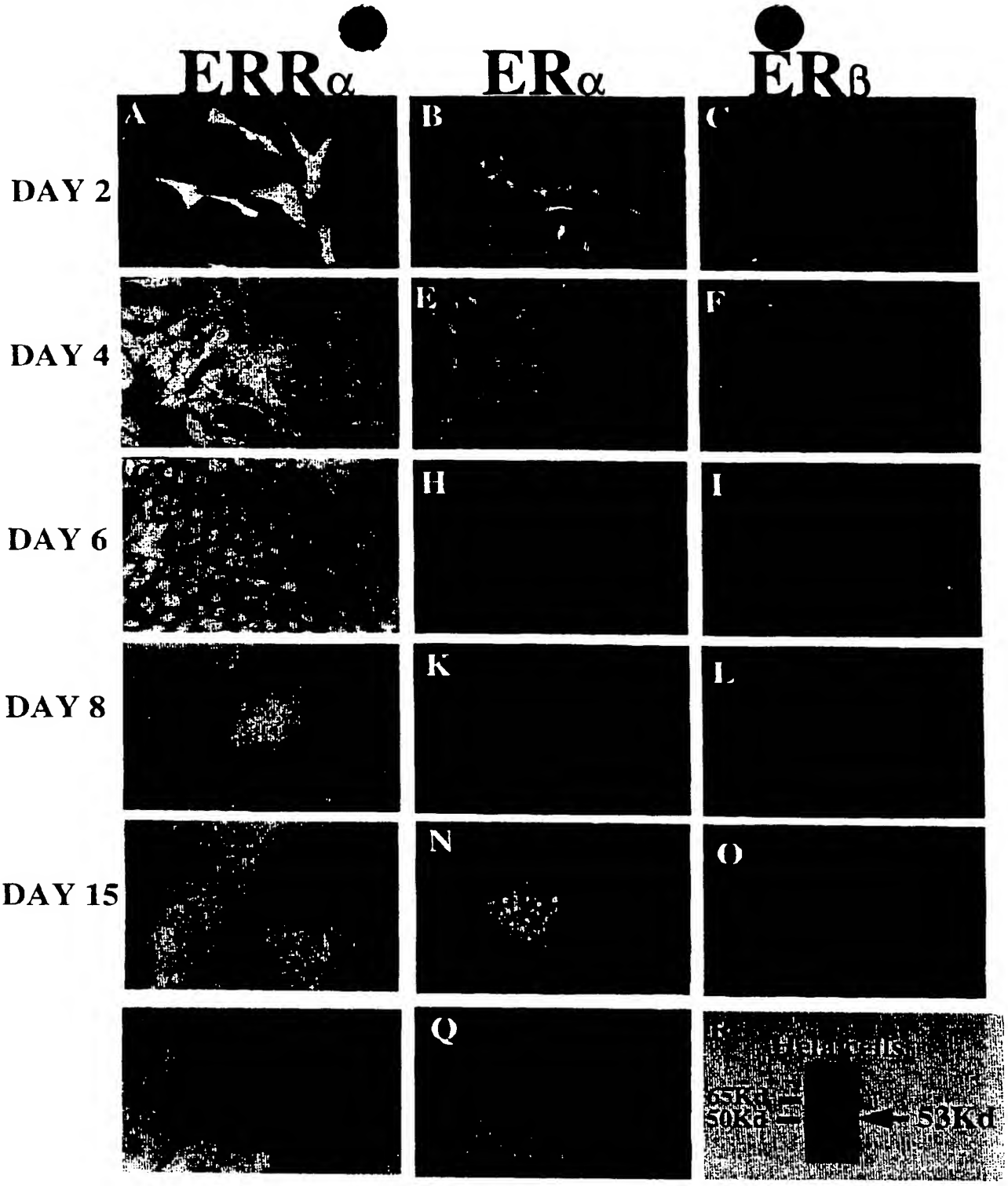

ERRα
ERα
ERβ
DAY 2
DAY 4
DAY 6
DAY 8
DAY 15
A
B
C
E
F
H
I
K
L
N
O
Q
R
50Kd
58Kd

Figure 4

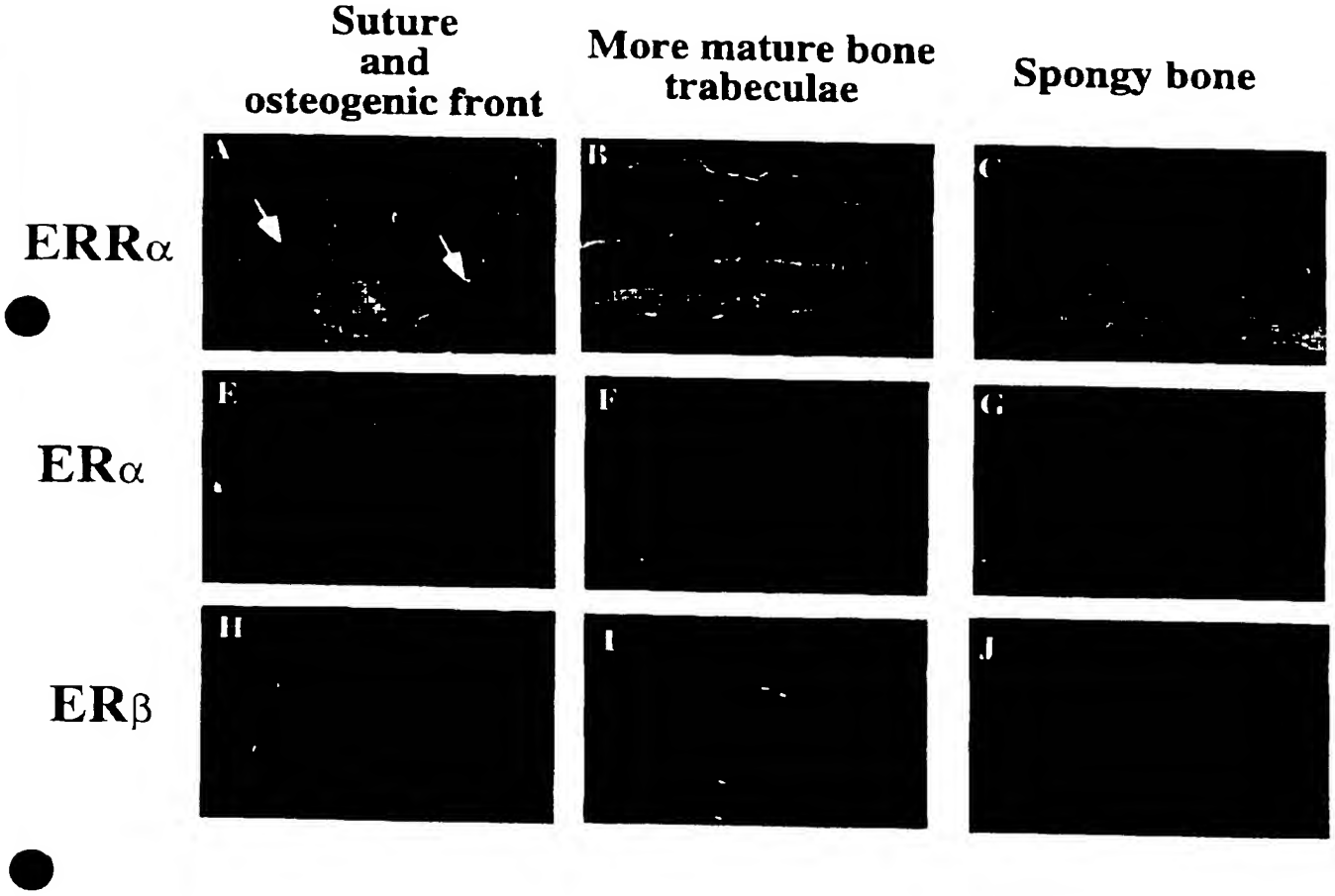
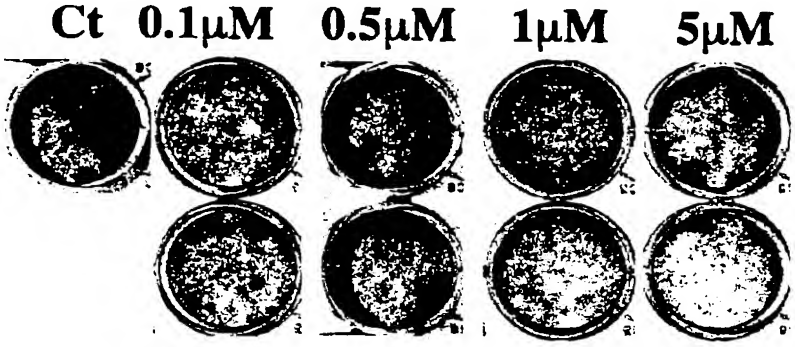

Suture and osteogenic front
More mature bone trabeculae
Spongy bone
ERRα
ERα
ERβ
A
B
C
E
F
G
H
I
J

Figure 5


Ct
0.1μM
0.5μM
1μM
5μM
SENSE
ANTISENSE
(AS)

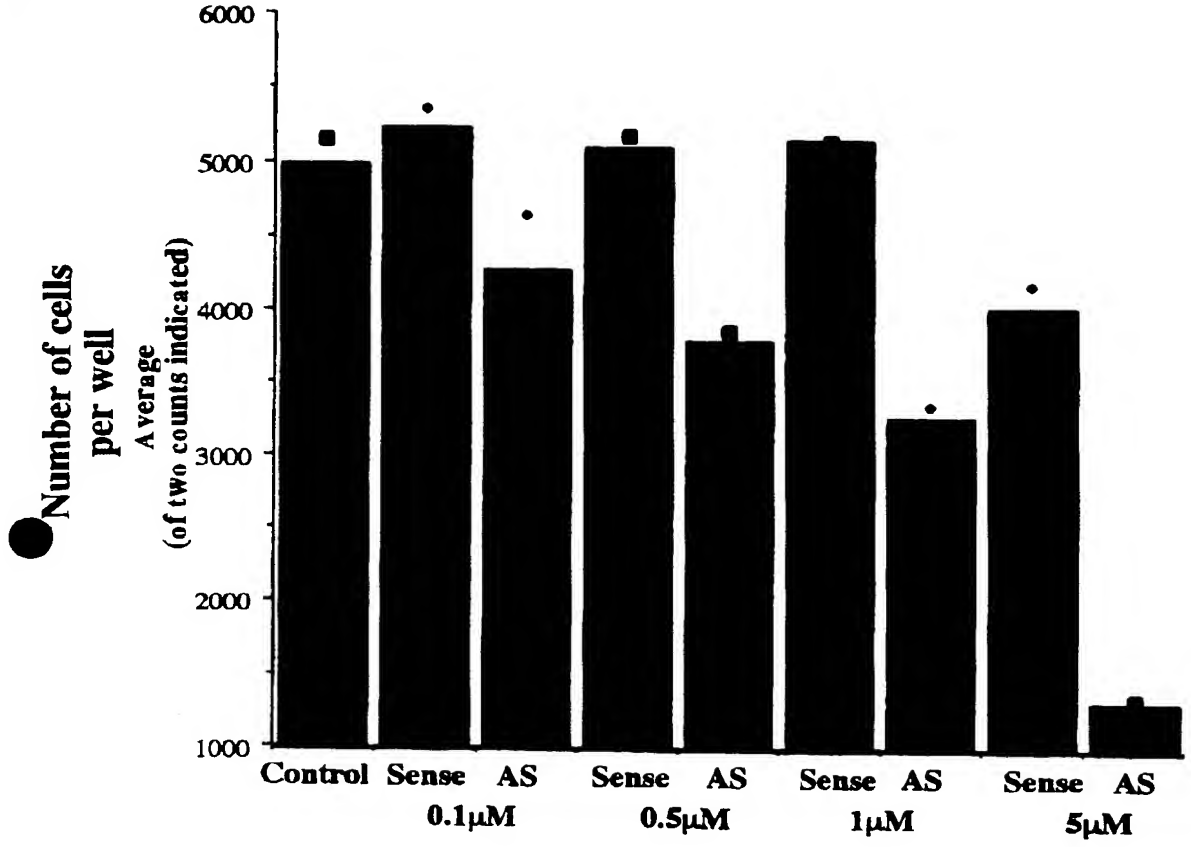
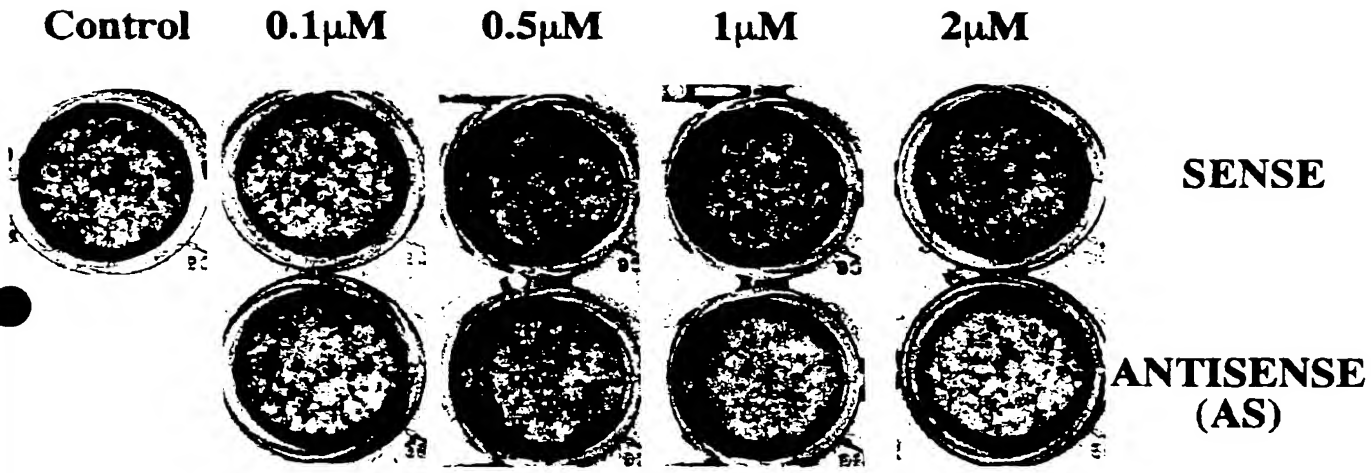

Number of cells per well
Average
(of two counts indicated)
6000
5000
4000
3000
2000
1000
Control
Sense
AS
0.1μM
Sense
AS
0.5μM
Sense
AS
1μM
Sense
AS
5μM

Figure 6


Control
0.1μM
0.5μM
1μM
2μM
SENSE
ANTISENSE
(AS)

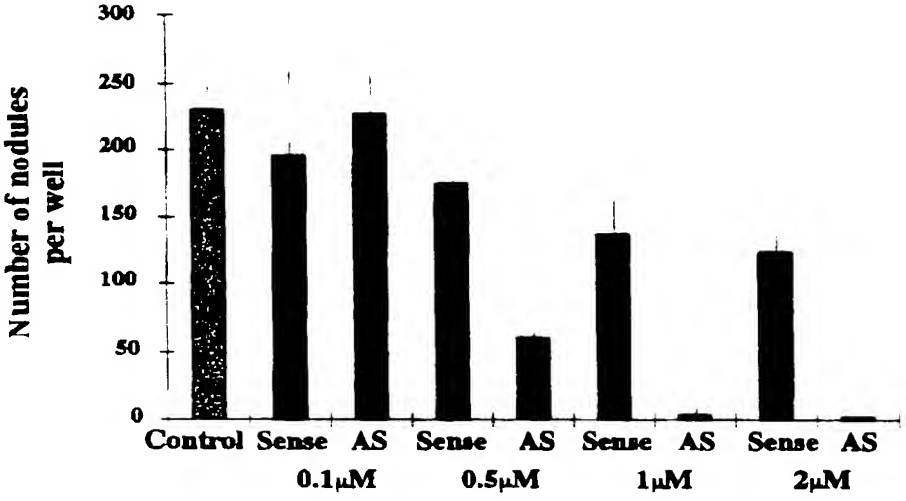

Number of nodules per well
300
250
200
150
100
50
0
Control
Sense
AS
Sense
AS
Sense
AS
Sense
AS
0.1μM
0.5μM
1μM
2μM

Figure 7

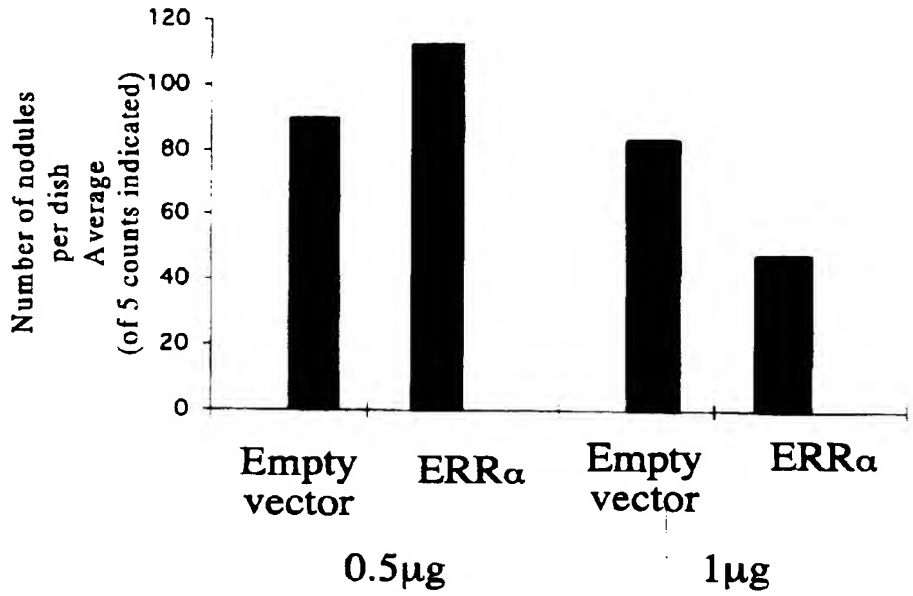
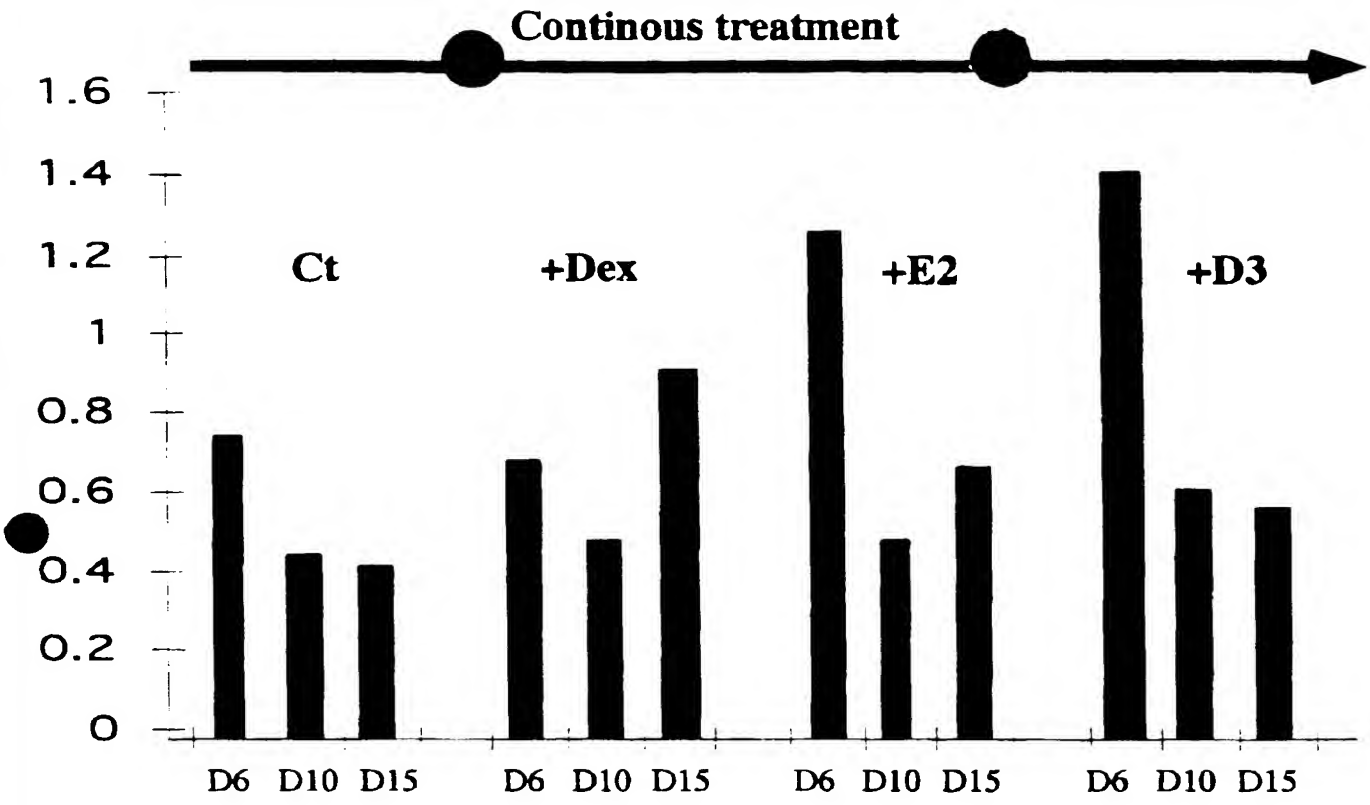

Number of nodules per dish Average (of 5 counts indicated)
120
100
80
60
40
20
0
Empty vector
ERRα
Empty vector
ERRα
0.5µg
1µg

Figure 8


Continous treatment
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ct
+Dex
+E2
+D3
D6 D10 D15
D6 D10 D15
D6 D10 D15
D6 D10 D15

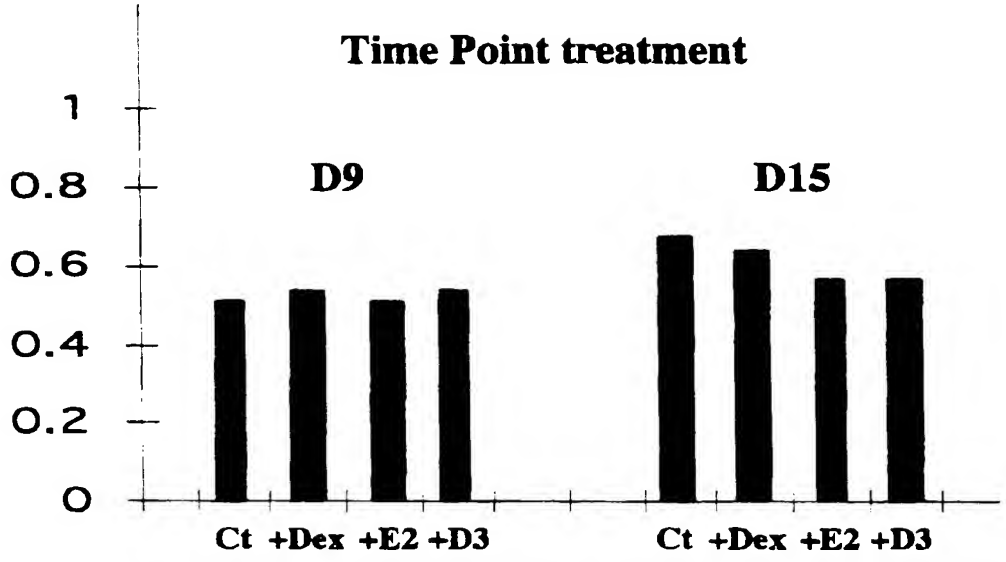

Time Point treatment
1
0.8
0.6
0.4
0.2
0
D9
D15
Ct +Dex +E2 +D3
Ct +Dex +E2 +D3

Figure 9